THE EFFECTS OF HEMOLYTIC STREPTOCOCCI ON THE BLOOD AND HEMOPOIETIC ORGANS OF RABBITS

PLATES 1 AND 2

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The present study was undertaken in order to throw further light, if possible, on the changes that may be caused by hemolytic streptococci on the blood and hemopoietic organs of rabbits.

In rabbits, the lymphocytes and basophile leukocytes occur in a higher percentage than in man. The granules of leukocytes stain with both basic and acid dyes and are highly refractive, and cells containing such granules are known as amphophiles and they correspond to the neutrophiles in man. The total number of leukocytes in the blood of normal rabbits as estimated by Brinkerhoff and Tyzzer [1] was from 6,400 to 13,400 per c mm. My counts in 10 normal rabbits average 9,600 leukocytes and 4,682,000 erythrocytes. The percentages of different types of leukocytes obtained by various observers are given in table 1.

TABLE 1

PERCENTAGES OF LEUKOCYTES OBTAINED BY VARIOUS OBSERVERS

	Brinkerhoff and Tyzzer,[2] Percentage	Bunting,[3] Percentage	The Author, Percentage
Amphophiles	40–50	53.5	40
Basophiles	4–8	8.8	6
Eosinophiles	0.5–1	0.5	0.5
Lymphocytes	45–55	53.5	48
Large mononuclears	2–8	7.1	5.5

It is unnecessary to deal with the structure of the marrow of rabbits in detail at this time as Muir,[4] Dickson,[5] and Brinkerhoff and Tyzzer [6] have described it fully. Normally the marrow of rabbits is more cellular than that of man and besides fat cells and supporting tissue it

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1 Jour. Med. Research, 1902, 7, p. 191.
2 Ibid., p. 173.
3 Univ. of Penn. Med. Bull., 1903, 16, p. 200.
4 Jour. Path. & Bacteriol., 1901, 7, p. 161
5 The Bone Marrow, a Cytological Study, 1908.
6 Jour. Med. Research, 1903, 8, p. 449.

consists of normoblasts, megaloblasts, myeoblasts, myelocytes of various types and megakaryocytes. The granules of the myelocytes are amphophile.

The structure of the spleen and lymph nodes does not differ much from that of man except, as Ehrlich [7] noticed, nucleated erythrocytes may occur in the spleen, hence this organ in the rabbit may form erythrocytes during postembryonic life.

Rabbits in good condition and as nearly as possible of the same age and weight were inoculated intravenously with varying amounts of 24-hour dextrose broth cultures of typical hemolytic streptococci (Beta type). The experiments were repeated two or three times in every case in order to secure reliable results. The blood was examined at least once before injection, twice within the first 8 hours after, and thereafter once or twice a day as condition might demand. In making the differential counts, 500 leukocytes were counted. The red cells were counted by means of the Levy hemocytometer and the hemoglobin estimated by the Dare method. The number of nucleated erythrocytes and of degenerated leukocytes was estimated by the number seen in counting 500 leukocytes. The condition of the erythrocytes was studied according to Schleip's [8] method: A drop of blood is diluted with physiologic salt solution and then allowed to run in between two cover glasses, one larger than the other; cedar oil or vaseline is applied around the edge of the hollow of a hanging drop slide, and the cover glasses placed in such a position that the small one lies just inside of the hollow and the larger in a perfect contact with the oil. By this means mechanical injury to the cell may be avoided. The dried blood films were stained with various methods, but the best result was obtained with Jenner's stain. The marrow, spleen and lymph nodes were fixed in Zenker's fluid, embedded in paraffin, and sections, five microns thick, stained with hemotoxylin and eosin or Jenner's blood stain. Marrow films were stained by the Mallory and Wright methods.[9]

The following experiments will serve to illustrate the results obtained and the method of procedure.

Exper. 1.—Rabbit A received 3 c c of culture of a hemolytic streptococcus isolated from the normal throat and rabbit B the same amount of culture of a strain from the throat of a scarlet fever patient. Both animals were killed on the tenth day. The blood picture showed no special features except rabbit B had a high percentage of amphophile leukocytes with slender ribbon-like nuclei in the form of a curve, the significance of which will be considered later.

[7] Quoted by Brinkerhoff, Ibid., p. 446.
[8] Atlas of Haematology, p. 10.
[9] Pathological Technique, 1918, p. 153.

TABLE 2
EXPER. 1, RABBIT B

	Total Number Leukocytes	Percentage							
		Amphophiles	Basophiles	Eosinophiles	Lymphocytes	Large Mononuclears	Transitionals	Myelocytes	Degenerated Amphophiles
Before inoculation	12,420	42.0	5.6	1.4	49.0	2.0	0	0	0
4 hours after	8,400	27.4	4.0	0	64.6	4.0	0	0	0
8 hours after	3,500	28.6	2.4	0	60.2	8.8	0	0	0
24 hours after	19,200	75.0	0	0	19.2	5.8	0	0	0
48 hours after	21,620	78.4	0	0	20.0	1.6	0	0	0
72 hours after	18,400	76.0	0	0	16.8	2.2	5.0	0	0
96 hours after	16,200	65.4	0	0	23.0	4.6	7.0	0	0
5 days after	19,300	72.6	2.0	0	15.0	6.2	4.2	0	0
6 days after	17,975	65.4	3.2	0	20.4	6.4	4.6	0	0
7 days after	23,075	66.2	2.0	0	12.4	9.4	10.0	0	0
8 days after	17,600	59.2	5.0	0	32.0	3.8	0	0	0
9 days after	14,550	44.4	4.0	0	39.4	2.2	0	0	0

Exper. 2.—The rabbit received 3 c c of broth culture of a streptococcus strain isolated from a case of cerebrospinal meningitis. The animal died with leukopenia associated with the presence of myelocytes. Degenerated leukocytes were found on the second day of infection.

TABLE 3
EXPER. 2

	Total Number Leukocytes	Percentage							
		Amphophiles	Basophiles	Eosinophiles	Lymphocytes	Large Mononuclears	Transitionals	Myelocytes	Degenerated Amphophiles
Before inoculation	1,065	39.2	6.0	0	48.0	6.8	0	0	0
4 hours after	4,425	24.0	5.6	0	64.4	6.0	0	0	0
8 hours after	5,640	21.4	6.2	0	66.8	5.6	0	0	0
24 hours after	21,240	76.2	0.2	0.4	18.6	0.4	4.2	0	5.3
48 hours after	25,400	75.4	2.0	0	10.0	8.0	6.6	0	8.2
72 hours after	3,125	26.6	0.8	0	41.0	9.2	4.0	6.4	3.0

Exper. 3.—A rabbit received 3 c c of culture of a streptococcus strain from the marrow of the rabbit in exper. 2. The blood picture of this animal is similar to the one in exper. 2, except that the percentage of degenerated leukocytes was much higher.

TABLE 4
EXPER. 3

	Total Number Leukocytes	Percentage							
		Amphophiles	Basophiles	Eosinophiles	Lymphocytes	Large Mononuclears	Transitionals	Myelocytes	Degenerated Amphophiles
Before inoculation	9,460	50.0	6.0	0	42.6	1.4	0	0	0
4 hours after	4,220	34.0	4.2	0	50.8	10.0	0	0	0
8 hours after	6,250	36.0	1.4	0	52.6	10.0	0	0	0
24 hours after	19,420	72.4	4.6	0	14.2	8.8	0	0	7.2
48 hours after	27,450	82.6	4.4	0	8.2	1.8	3.0	0	11.2
72 hours after	15,200	50.2	0.8	0.2	20.4	14.2	7.4	5.0	13.3
96 hours after	7,625	32.4	2.4	0	46.2	6.0	5.2	8.8	9.5

Exper. 4.—A rabbit received 3 c c of culture of a streptococcus strain from a case of bronchopneumonia after it had been passed through mice 16 times. The degeneration of leukocytes was most marked on the third day and leukopenia developed on the day the animal died.

TABLE 5
EXPER. 4

	Total Number Leukocytes	Percentage							
		Amphophiles	Basophiles	Eosinophiles	Lymphocytes	Large Mononuclears	Transitionals	Myelocytes	Degenerated Amphophiles
Before inoculation	11,040	42.2	6.8	0	50.4	0.6	0	0	0
4 hours after	7,460	29.0	1.0	0	54.6	15.4	0	0	0
8 hours after	3,600	28.2	0.8	0	59.4	11.6	0	0	0
24 hours after	21,200	68.2	0.8	0	22.8	8.2	0	0	8.2
48 hours after	24,040	74.0	2.0	0	19.4	4.6	0	0	11.7
72 hours after	16,200	42.4	2.4	0	24.6	7.2	9.2	14.2	18.3
96 hours after	6,425	29.0	5.4	0	35.0	9.2	5.8	15.6	8.4

TABLE 6
EXPER. 5

	Total Number Leukocytes	Percentage							
		Amphophiles	Basophiles	Eosinophiles	Lymphocytes	Large Mononuclears	Transitionals	Myelocytes	Degenerated Amphophiles
Before inoculation	11,400	37.8	4.4	0.6	47.2	10.0	0	0	0
4 hours after	9,250	29.0	1.0	0	64.2	5.8	0	0	0
8 hours after	4,265	22.0	4.0	0	61.0	13.0	0	0	0
24 hours after	14,200	57.0	4.0	0	24.0	9.6	5.4	0	27.5
48 hours after	1,040	47.2	6.0	0	23.8	9.2	9.8	4.0	25.2
72 hours after	4,250	34.6	4.2	0	48.4	4.8	0.6	7.4	20.5

TABLE 7
EXPER. 6

	Total Number Leukocytes	Percentage							
		Amphophiles	Basophiles	Eosinophiles	Lymphocytes	Large Mononuclears	Transitionals	Myelocytes	Degenerated Amphophiles
Before inoculation	10,620	45.0	4.0	0	47.2	3.8	0	0	0
4 hours after	4,725	21.0	0	0	69.4	9.6	0	0	0
8 hours after	5,675	27.2	0	0	59.6	13.2	0	0	0
24 hours after	14,825	60.2	2.0	0	22.2	5.6	0	0	0
48 hours after	18,020	52.8	4.0	0	35.2	8.0	0	0	0
72 hours after	17,400	60.6	5.2	0	26.2	8.0	0	0	0
96 hours after	16,325	57.0	2.0	0	32.8	5.2	3.0	0	0
5 days after	13,000	54.0	4.8	0	32.0	3.0	6.2	0	0
6 days after	14,025	56.2	6.4	0	26.6	6.0	4.8	0	0
7 days after	21,050	68.2	3.0	0	21.6	2.2	5.0	0	0
8 days after	25,625	78.4	1.0	0	19.0	1.0	0.6	0	0
9 days after	24,040	81.0	0	0	16.8	0.2	2.0	0	0
10 days after	22,040	72.4	0	0	23.0	0.6	4.0	0	0
11 days after	16,675	72.6	2.0	0	15.0	3.0	7.4	0	0
12 days after	15,940	65.0	0	0	29.2	2.2	3.6	0	0
13 days after	26.000	76.2	2.0	0	12.4	3.0	2.4	4.0	4.2
14 days after	14,500	65.4	3.2	0	10.4	5.0	2.8	13.2	0
15 days after	8,842	46.2	2.0	0	32.4	2.4	5.0	12.0	0

Exper. 5.—A rabbit received 3 c c of culture of a streptococcus strain from the heart of a woman who died of puerperal sepsis. The largest number of leukocytes was 14,200 and the degeneration of leukocytes was more marked than in any other case.

Exper. 6.—A rabbit received 1 c c of a virulent streptococcus culture every 6 days; the animal died on the 16th day. The blood smears showed no degenerated leukocytes and myelocytes appeared in the peripheral circulation on the 13th day, but the former disappeared on the following day.

Exper. 7.—A series of 5 rabbits were inoculated with nonvirulent hemolytic streptococci in small doses every 6 days, and by this method I was able to produce a condition of anemia in two, one (C) giving 2,844,090 erythrocytes and 26% of hemoglobin, and the other (D) 3,046,000 erythrocytes and 28% of hemoglobin. Anisocytosis appeared earlier than poikilocytosis which was first noted at the end of the 4th week in rabbit C. The normoblasts as a rule appeared in the peripheral circulation as early as on the 2nd week after injection, but their number never was above 50 in counting 500 leukocytes. The erythrocytes of both animals after the 5th week showed diminished and uneven hemoglobin distribution and polychromatophilia, some of the cells containing minute black deposits at the periphery. No megaloblasts were found in the blood films. The marrow showed hyperplasia of normoblasts, and otherwise it was normal. The spleen showed a marked congestion and a large number of phagocytic cells crowded with blood pigments.

DISCUSSION

Leukocytes.—The animals inoculated with fatal doses of virulent hemolytic streptococcus usually showed degenerative changes in the amphophile leukocytes on the second day. Careful study showed that the principal and foremost change occurred in the nuclei of the amphophile leukocytes, in the form of swelling and disintegration. In some cases fragmentation and condensation of the nuclei were found. The cytoplasm was also abnormal: There was early an increase in the size of the granules and of the cell body itself, most of the swollen leukocytes having only a few poorly stained granules on a pale or pink background. In exper. 2, vacuoles in the cytoplasm of the amphophile leukocytes were noted the day before death. Schleip [8] states that vacuolar degeneration of leukocytes may occur in much debilitated or moribund patients some hours before death, and this condition he regards as a bad prognostic sign. The largest number of degenerated leukocytes was found in exper. 5. This animal did not have a high leukocyte count.

Arneth [11] regards the nuclear segments as representing the age of the cells, and when cells with one or two segments become more numerous than normal ("deviation to the left"), it is supposed to signify an impending exhaustion of the marrow. He divides neu-

[11] Die neutrophilen weissen Blutkörperchen, Jena, 1904, p. 17.

trophile leukocytes into five classes according to the number of nuclear segments. Schilling [12] separates neutrophile leukocytes into segmented nuclear cells, rod-nuclear cells, juvenile cells, and myelocytes and concludes that deviation to the left may mean either regeneration or exhaustion of the marrow, and deviation to the right a degeneration of leukocytes. Nagao [13] found that the Arneth index became disarranged immediately after the injection of killed nonhemolytic streptococci in guinea-pigs, reaching its maximum in about 3 hours, and then gradually returning to normal. Schilling states that the number of nuclear segments has no relation to the age of the cells and Neumann [14] calls attention to the fact that polymorphonuclear leukocytes become simple when they ceased their ameboid movement. My study shows that the nuclei which were undergoing degeneration offer a good deal of difficulty in the recognition of nuclear segments. In experiments 1 and 6 the amphophile leukocytes with a slender ribbon-like nucleus in the form of a curve occurred in a higher percentage than in the other cases. These cells correspond morphologically to the cells with "tiefeinge-buchtetem Kerne" according to Arneth and an increase in the number of these cells should mean a deviation to the left and be a bad prognostic sign, but the experiment indicates the contrary. However, the appearance of myelocytes especially when associated with leukopenia seems to be a danger signal from the data thus far obtained. In view of what has been said, Schilling's deduction seems to be more valid, and clinical application of the original Arneth index should be made with reservation.

The Marrow.—In acute streptococcus infection Dickson found in the marrow hemorrhage and a large number of phagocytic cells which were crowded with pigments in one case, in another case there was a marked necrosis of the marrow cells. He also described "gelatinous degeneration" of the marrow in acute and chronic cases. As to the significance of this gelatinous degeneration which bears on the leukoblastic reaction, Muir [15] holds that this change may interfere with cellular hyperplasia. Lucibelli [16] found that loss of straining property, irregular distribution of chromatin, swelling and solution of the neuclei of the marrow cells followed fatal injection of typhoid, paratyphoid, and colon bacilli. Muir [17] found the same changes in experimental staphylococcus infection.

[12] Quoted by Gruner: The Biology of Blood-Cells, 1913, p. 217.
[13] Jour. Infect. Dis., 1920, 27, p. 22.
[14] Quoted by Gruner: The Biology of Blood Cells, 1913, p. 216.
[15] Trans of the Path. Soc., London, 1902, 53, p. 379.
[16] Quoted by Gruner, The Biology of Blood-Cells, 1913, p. 199.
[17] Jour. Path. & Bacteriol., 1901, 7, p. 161.

In my experiments hyperplasia in the marrow usually occurred after inoculation of streptococci regardless of the virulence or number of organisms inoculated. In the early stage of virulent streptococcus infection, the cytoplasm of myelocytes presented a granular appearance and stained more strongly with eosin. In general, the degenerative changes of the marrow cells resulting from infection with virulent hemolytic streptococci in rabbits consisted of karyorrhexis, karyolysis, vacuolation and solution of cytoplasm. The microphotographs give a better idea of the change than an extended verbal description would. In exper. 5 the marrow consisted mostly of myeloblasts, and all the leukoblastic cells were undergoing degeneration especially at the periphery of the marrow. In exper. 6 the marrow presented the gelatinous degeneration described by Dickson, and only a few marrow cells were seen here and there in a pink homogeneous material. A rabbit killed on the second day of virulent streptococcus infection had a similar change in the marrow, but the areas were small and consisted mostly of delicate febrils running in all directions, forming an interlacing network. Strange to say, this picture is practically identical to that described by Dickson in a case of ulcerative endocarditis of less than three weeks' duration.

The megakaryocytes seem to be very sensitive to toxic action as they undergo degeneration in rabbits inoculated with hemolytic streptococci of only slight virulence. In no case could the three protoplasmic zones be made out. The cytoplasm had a strong affinity for eosin, and the nuclei were swollen, fragmented, poor or rich in chromatin. These cells usually were present in large numbers within the first 48 hours after injection, and then gradually decreased in number as the course of infection advanced. Within them erythrocytes, myelocytes, and amphophile leukocytes were found. The phagocytic cells described by Dickson were noted in several cases, especially when there was hemorrhage in the marrow.

The Spleen.—Evans [18] showed that streptococci may produce acute splenic swelling of a gray type characterized by less congestion and hyperplasia of the cells of myeloid type, and he argues that there is no hyperplasia of the reticuloendothelial cells in contrast with the red type because the leukocytes then are still in function. Muir found a hyperplasia of phagocytic cells in spleen in experimental staphylococcus infection. Nagao [19] produced a marked degeneration of the cells in the malpigihian bodies by the injection of nonhemolytic streptococci.

[18] Johns Hopkins Hosp. Bull., 1916, 27, p. 356.
[19] Jour. Infect. Dis., 1920, 27, p. 22.

In exper. 2 the spleen presented a marked congestion and hemorrhage, and some of the erythrocytes were poor in hemoglobin and others appeared in different shapes. A large number of phagocytic cells were crowded with blood-pigments and Flemming's bodies and the latter were more numerous in exper. 5 than in any other case. By many Flemming's bodies are considered to be derived from degenerated lymphocytes, but after a careful study I believe that some of them come from degenerated polymorphonuclear leukocytes. Nucleated erythrocytes and myelocytes were found practically in every case. The Malpigihian bodies were usually decreased in size, and in exper. 3 a necrotic change ("focal necrosis") occurred in the center.

The lymph glands presented no special features. Congestion and slight hemorrhage were seen in certain acute cases and leukocytic infiltration occurred in every case. Foci of necrosis of the lymph follicles were found in exper. 3.

Leukocytosis and Leukopenia.—Leukocytosis and leukopenia have been explained on the basis of chemotaxis and reaction on part of the marrow. In leukocytosis the explanation rests on a good evidence, but the origin of leukopenia is still a matter of speculation, and its development, in spite of the presence of chemotactic substances, is hard to understand. The view that leukopenia is due to an exhaustion of the marrow seems reasonable and the question arises whether a normal marrow can become exhausted without a preliminary leukoblastic reaction. If not, what other possibilities are there that exhaustion of marrow that eventually can give rise to leukopenia may be produced? To answer this question it is necessary to study the condition of marrow as well as that of blood. During the course of my experiments two types of leukopenia were observed in severe infections: the one occurring immediately after inoculation and the other some time later. The former seems to be largely due to an abnormal distribution of leukocytes, while the latter probably is due to the retrogressive changes of amphophile leukocytes in the circulation and of marrow. This is illustrated especially by exper. 5.

Streptococcus Leukocidin.—Hektoen [20] found that the culture fluids of virulent streptococci may not only decrease the phagocytic activity of leukocytes, but also cause swelling and arrest of ameboid movement and grave morphologic changes, and M'Leod [21] noted a retrogressive change of the amphophile leukocytes in the heart blood of

[20] Jour. Am. Med. Assn., 1906, 46, p. 1407.
[21] Jour. Path. & Bacteriol., 1915, 19, p. 392.

rabbits that died of streptococcus infection. Ruediger [22] and Nakayama [23] brought to light the toxic effect of virulent streptocci on leukocytes by means of the methylene blue reduction test, leukocytes acted on by streptococcus filtrates failing to reduce the blue.

By the smear method I tested the leukocytal action of the strain of hemolytic streptococcus used in exper. 4. The organism was cultivated in serum broth for 24 hours, and the filtrate which had been passed through a Massen filter was used to test for leukocidin. The leukocytes were obtained from the sterile peritoneal exudate of a rabbit produced by injection of aleuronat suspension. The marrow was removed from the shaft of a femur and suspended in salt solution and by shaking a heavy suspension of marrow cells could be obtained. The result is illustrated in table 8.

TABLE 8

LEUKOCYTAL ACTION OF STRAIN OF HEMOLYTIC STREPTOCOCCUS USED IN EXPER. 4

Amount of Culture Filtrate	Leukocytes	Result	Marrow	Result
1 c c	1 c c	+	1 c c	+
0.75 c c	1 c c	+	1 c c	+
0.5 c c	1 c c	+	1 c c	+
0.25 c c	1 c c	0	1 c c	+
0.1 c c	1 c c	0	1 c c	0
Serum broth	1 c c	0	1 c c	0
Salt solution	1 c c	0	1 c c	0

As a rule, after one hour's incubation, at 37C, of the mixture of leukocytic suspension and streptococcus filtrate the amphophile leukocytes became swollen, the cytoplasm granular, staining with methylene blue, and in certain cases undergoing solution. The nuclei also became swollen and occasionally fragmented. In control specimens containing serum broth or salt solution the leukocytes remained normal as long as three hours. The streptococcus filtrate practically exerted the same effect on marrow cells in suspension, but the degenerative changes appeared much earlier. The changes resembled altogether those that occurred in vivo. It is noteworthy that culture filtrates injected intravenously by me did not cause any toxic effect on the leukocytes in the peripheral circulation and only a slight hyperplasia of marrow. This may be because the toxin was not concentrated enough to produce a massive effect, and because degenerated leukocytes, if any were produced, were taken out of the peripheral circulation promptly.

[22] Jour. Am. Med. Assn., 1905, 44, p. 198.
[23] Jour. Infect. Dis., 1920, 27, p. 86.

CONCLUSIONS

Hemolytic streptococci may produce a toxic substance that causes degeneration of leukocytes and marrow cells in vitro.

In rabbits intravenous injections of virulent hemolytic streptococci produce retrogressive changes in the amphophile leukocytes in the peripheral circulation and in the marrow cells. This change is probably due to the same substance that acts on the cells in vitro, but of course the disintegraated products of streptococci may have the same effect. This degeneration in amphophile leukocytes and marrow cells is the factor that may give rise to leukopenia.

Hemolytic streptococci of low virulence produce no definite morphologic changes in the amphophile leukocytes in vitro or vivo, but cause a disarrangement of the Arneth index to the left.

Hemolytic streptococci may produce gelatinous degeneration of marrow.

Hyperplasia of phagocytic cells in spleen may take place in hemolytic streptococcus infection.

By subacute infection with small doses of nonvirulent hemolytic streptococci, a condition of advanced anemia may be produced (exper. 7). Since there is a lower hemoglobin content, an absence of megaloblasts and no marked lesions in the marrow, the anemia is of secondary type.

EXPLANATION OF PLATES 1 AND 2

Fig. 1.—Rabbit leukocytes after being acted on by culture filtrate of virulent hemolytic streptococcus; X 1000.

Fig. 2.—Rabbit leukocytes acted on by virulent streptococcus culture; X 1000.

Fig. 3.—Blood smear on the second day of infection showing degenerated amphophiles; exper. 5, Jenner's stain; X 1000.

Fig. 4.—Marrow smear, exper. 3, showing degenerated marrow cells; X 1000.

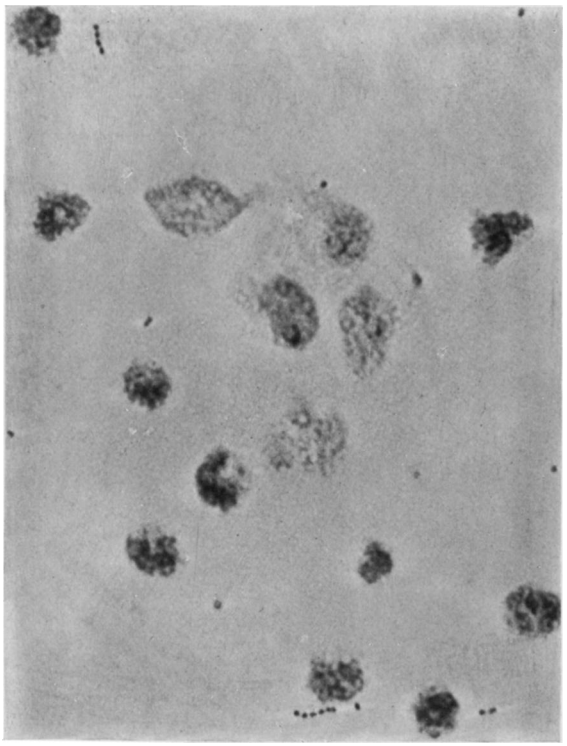
Fig. 5.—Section of marrow, exper. 5, showing marked degeneration; X 1000.

Fig. 6.—Gelatinous degeneration of marrow in early stages of acute infection; exper. 3; X 1000.

Fig. 7.—Advanced gelatinous degeneration of marrow; exper. 6; X 500.

Fig. 8.—Section of spleen showing several phagocytic cells crowded with erythrocytes and black deposits; exper. 6; X 1000.

PLATE 1



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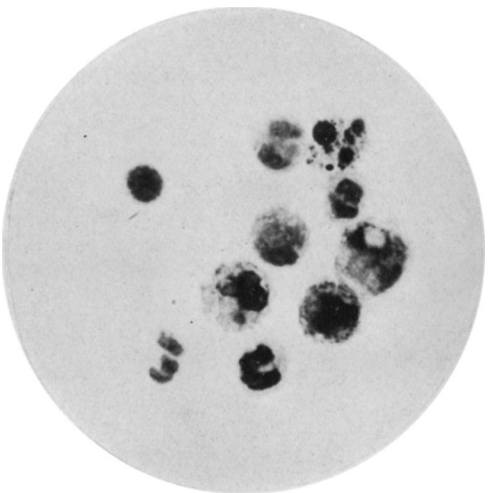

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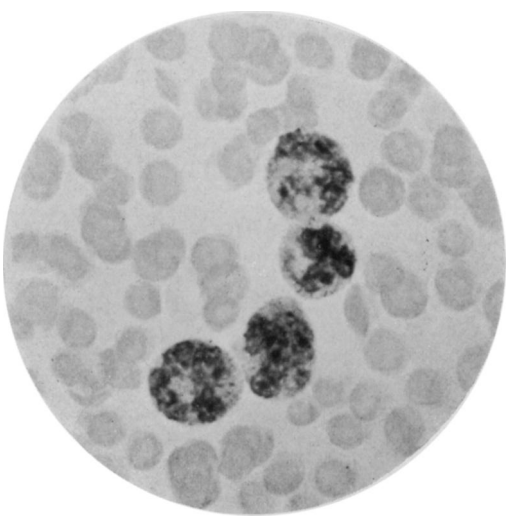

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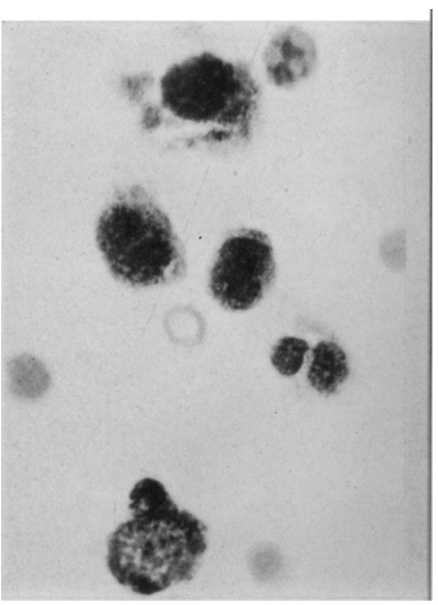

PLATE 2